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Angiotensin III up-regulates genes involved in kidney damage in mesangial cells and renal interstitial fibroblasts

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Angiotensin III up-regulates genes involved in kidney damage in mesangial cells and renal interstitial fibroblasts. Angiotensin (Ang) II is considered the effector peptide of the renin-angiotensin system (RAS) that acts as a renal growth factor. Some studies have shown that the angiotensin degradation product Ang III presents some biological activities, though its role in renal pathology has not been explored. We have observed that in renal interstitial fibroblasts Ang III induces c-*fos* gene expression, suggesting a potential role of Ang III in the control of cell proliferation. To study the involvement of Ang III in matrix regulation, we determined whether Ang III increased TGF-β gene expression and fibronectin production in cultured rat mesangial cells and renal interstitial fibroblasts, the main effector cells in glomerular and interstitial fibrosis, respectively. In both cell types, treatment with Ang III (10^{-7} M) for six hours up-regulated gene expression of transforming growth factor-β1 (TGF-β1; 2.3- and 2.2-fold, respectively). This peptide also increased fibronectin production in renal interstitial fibroblasts. All these data suggest that Ang III could contribute to matrix accumulation. Activation of local RAS has been described during renal damage. Renal cells express angiotensinogen mRNA that was up-regulated in response to Ang II and Ang III stimulation, and therefore both peptides may participate in the generation of angiotensin peptides in the kidney. In conclusion, our results suggest that the angiotensin degradation product Ang III could participate in the pathogenesis of key events of renal diseases, supporting the hypothesis that other peptides of the RAS besides Ang II may be involved in renal injury.

Angiotensin (Ang) II is a powerful vasoconstrictor agent that participates in local and systemic hemodynamic regulation. In the last few years, Ang II has been recognized as a growth factor that plays an active role in renal pathology [reviewed in 1, 2]. Although Ang II has been considered the effector peptide of the renin-angiotensin system (RAS), recent studies suggest that other angiotensin peptides are bioactive agents [reviewed in 3]. Ang III presents some physiological functions similar to Ang II in cardiovascular and central nervous systems, though in some of them, such as the pressor response, Ang III is less potent than Ang II [3]. Renal infusion of Ang II and Ang III increased the fractional clearance of albumin [4]. Ang (1-7) presents some opposite effects to Ang II, acts as a vasodilator agent and inhibits vascular smooth muscle cells growth [3]. However, the potential role of Ang degradation products in renal pathology is unclear. In addition, some of Ang II actions seem to be due to these degradation peptides. Thus, the release of vasopressin requires the conversion of Ang II to Ang III [5], and the plasminogen activator inhibitor-1 expression induced by Ang II is mediated by Ang IV through the angiotensin II receptor type-4 (AT_4) [6].

The kidney possesses all of the machinery necessary to generate and degrade the angiotensin peptides. Ang II can be converted to Ang III by aminopeptidase A (APA) present in glomeruli and mainly in tubuli [review in 3]. In the lumen of proximal tubular cells, the concentration of Ang II is 1000 times higher than in plasma, but due to the high density of peptidases, it only represents around 5 to 15% of total renal angiotensins [7]. In pathological settings, the concentration of proteolytic enzymes increases due to the activation of resident and infiltrating cells. On the other hand, elevated renal APA mRNA expression has been found in the glomeruli of animals with several models of renal injury [8, 9]. All these data support the idea of the presence of angiotensin degradation products in the kidney in normal and pathological conditions.

The main features of renal damage are proliferation of resident cells, excessive accumulation of extracellular matrix and mononuclear cell recruitment [10]. Some authors, including ourselves, have demonstrated that Ang II participates in all of these phenomena, stimulating directly renal cells to proliferate (mainly mesangial cells and interstitial fibroblasts), to increase matrix production and to synthesize chemotactic factors [1, 2, 11–13]. In addition, the *in vivo* blockade of Ang II actions by treatment with angiotensin converting enzyme inhibitors and angiotensin receptor antagonists decreased the renal lesions and inflammatory infiltration in several models of renal injury [14–17]. All these data, together with the fact that in pathological conditions an increased local Ang II generation exists as

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well as general and specific proteases activation [3], strongly suggest the presence of Ang II degradation products with the potential to renal damage activity.

The aim of this work is to test the hypothesis that other peptides of the RAS, such as the degradation product Ang III, may also participate in the pathogenesis of some key events associated with the progression of renal damage. For this reason, we studied whether Ang III regulates cell growth and matrix production, determining whether this peptide presents similar or opposing properties to those of Ang II on such phenomena.

METHODS

Cell cultures

Rat mesangial cells were cultured by several sieving techniques and characterized as previously described [13]. The NRK 49F cell line (ATCC:CRL1570; American Type Culture Collection, Rockville, MD, USA) is derived from rat kidney fibroblasts. Cells were grown in DEMEN with 5% FCS. At confluence, cells grown in 75 cm^2 flasks were made quiescent for 48 hours in serum-free medium, and then different studies were performed.

Northern blot and polymerase chain reaction

The expression of transcripts for c-*fos* and TGF-β1 was determined by Northern blot analysis, while angiotensinogen (Ao) gene expression was studied by a semiquantitative RT-PCR technique as previously described [12]. Briefly, PCR analysis for angiotensinogen and glyceraldehyde 3′-phosphate dehydrogenase (G3PDH) was conducted in the following conditions (1 min at 63°/54°C, respectively, 3 min at 72°C and 1 min at 94°C, 25 cycles). The primers used for rat angiotensinogen were: (antisense) 5′-CCAGCCGGGAGGTGCAGT-3′and (sense) 5′-TTCAGGCCAAGACCTCCC-3′, and for G3PDH: (antisense) 5′-ATACTGTTACTTATACCGATG-3′ and (sense) 5′-AATGCATCCTGCACCACCAA-3′ that yielded products of 308 and 515 bp, respectively. In all experiments control reactions were done to check for the presence of contaminants. The DNA products were analyzed on 4% polyacrylamide/urea gels. The gels were dried and exposed to X-ray films.

Determination of fibronectin synthesis

New fibronectin (FN) synthesis was measured by metabolic labeling with [^{35}S]-methionine and immunoprecipitation with anti-FN antibodies, as previously described [12]. Briefly, quiescent cells were incubated for 24 hours with Ang II and Ang III in methionine-free culture medium RPMI with 20 μCi/ml of [^{35}S]-methionine. Then, supernatants were immunoprecipitated with an excess of anti-FN antibody (50 μg) for 16 to 18 hours at 4°C, recovered with protein A-Sepharose beads (Pharmacia, Uppsala, Sweden), and were analyzed by PAGE-SDS. FN production was normalized to DNA content. As a negative control for immunoprecipitation, isotopic normal rabbit IgG (30 μg/ml) was used. Recombinant human TGF-β (50 pM) was employed as positive control for FN synthesis.

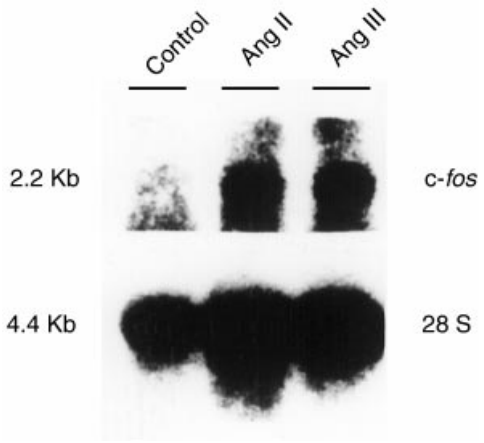

Fig. 1. Angiotensin (Ang) III induces c-*fos* mRNA expression in renal interstitial fibroblasts. Cells were incubated with 10^{-7} M Ang III and Ang II for one hour. Then RNA was extracted and c-*fos* gene expression was determined by Northern blot. A representative experiment of two is shown.

Statistical analysis

Results are expressed as the mean ± SEM Significance was established using Student's *t*-test and analysis of variance. Differences were considered significant if the *P* value was less than 0.05. Autoradiograms were scanned using the Image Quant densitometer (Molecular Dynamics, Sunnyvale, CA, USA) and results were expressed as arbitrary densitometric units relative to 28S intensity bands (Northern blot) and G3PDH (RT-PCR).

RESULTS

Ang III induces the expression of growth-related nuclear proto-oncogene c-*fos*

To investigate whether Ang III could play a potential role in the control of cell growth we studied the gene expression of c-*fos*, an early response gene that has been associated with cell proliferation, cellular differentiation, and hypertrophy [18]. Quiescent renal interstitial fibroblasts were stimulated with 10^{-7} M Ang II and Ang III for one hour. Then, RNA was isolated and c-*fos* mRNA expression analyzed by Northern blot. Ang III stimulation induced c-*fos* gene expression, similar to Ang II, while this gene did not appear in control cells (Fig. 1).

Ang III elicits up-regulation of TGF-β gene expression and fibronectin synthesis

To determine whether Ang III could participate in matrix regulation we evaluated the effects of Ang III on TGF-β gene expression, the major cytokine involved in matrix regulation [19], and on fibronectin synthesis, a representative matrix protein that is synthesized early

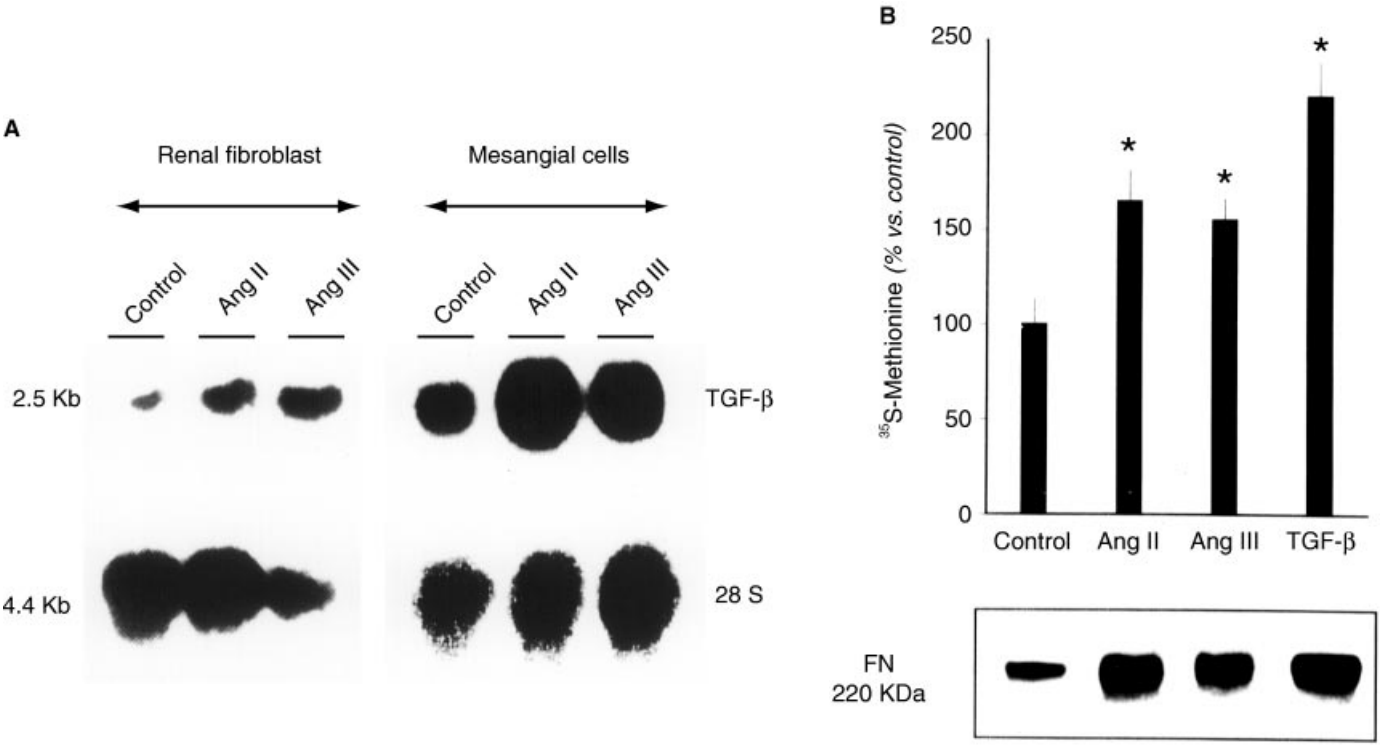

Fig. 2. Ang III increases TGF-β1 mRNA expression (*A*) and fibronectin (FN) synthesis (*B*). (A) Cells were stimulated with 10^{-7} M Ang III and Ang II for six hours. A representative Northern blot of two is shown. *(B)* Renal interstitial fibroblasts were metabolically labeled with ^{35}S-methionine and stimulated with 10^{-7} M Ang III and Ang II, and 50 pM TGF-β for 24 hours. Figure shows the densitometric analysis of the FN-bands in the upper part and in the lower part shows a representative autoradiography of PAGE-SDS of FN. Data were shown as percentage of increase versus control in arbitrary units. Mean ± SEM of 3 experiments. $^*P < 0.05$ versus control.

during renal damage, preceding collagen deposition and fiber formation [20]. Mesangial cells and interstitial fibroblasts are the main cells involved in glomerular and interstitial fibrosis [10, 21], respectively, and thus we chose them for our studies.

Cultured mesangial cells and renal interstitial fibroblasts (NRK 49F) were growth arrested by serum depletion for 48 hours. Cells were incubated in serum-free medium with Ang III and Ang II (10^{-7} to 10^{-11} M) for six hours. RNA was then extracted and gene expression was determined by Northern blot. In renal interstitial fibroblasts, we have previously shown that Ang II increased the mRNA levels of TGF-β1 with a maximal peak at six hours of incubation [12]. In those cells, Ang III up-regulated TGF-β gene expression with a kinetics and intensity similar to Ang II, being maximal with 10^{-7} M Ang III after six hours of incubation (2.2-fold vs. control; Fig. 2A). Exposure of cultured rat mesangial cells to 10^{-7} M Ang III for six hours also up-regulated TGF-β1 mRNA levels (2.3-fold) similar to that observed with Ang II (Fig. 2A).

To determine the Ang III effect on fibronectin production, metabolic labeling and immunoprecipitation were done. Treatment of resting fibroblasts with Ang II and Ang III (10^{-7} to 10^{-11} M) for 24 hours caused a significant increase in fibronectin synthesis, with a maximal response at 10^{-7} M Ang III (155 ± 14% increase vs. control (100 ± 11, $N = 3$; $P < 0.05$), similar to that observed with Ang II (165 ± 12% increase vs. control, $N = 3$, $P < 0.05$; Fig. 2B). In some experiments TGF-β (50 pM) was employed as a positive control (220 ± 18% increase vs. control, $N = 3$, $P < 0.05$). All these data suggest that Ang III, through TGF-β gene expression and fibronectin production, could participate in matrix accumulation during renal injury.

Renal cells express angiotensinogen mRNA levels: Regulation by Ang III and Ang II

Recent studies have shown that renal tubular cells and interstitial fibroblasts express angiotensinogen mRNA [2, 12]. This gene is also expressed by mesangial cells (Fig. 3), suggesting that all these cells could contribute to the generation of angiotensin peptides in the kidney. Ang II up-regulates angiotensinogen gene expression in the liver, in cardiac myocytes and in renal interstitial fibroblasts [12, 22]. In cultured mesangial cells, Ang II and Ang III increased angiotensinogen mRNA expression (2.7- and 2.8-fold, respectively at 10^{-7} M after 6 hr; Fig. 3). Similar results were observed in renal interstitial fibroblasts (Fig. 3). These results suggest that those peptides could contribute to an increase in the renal generation of angiotensin peptides.

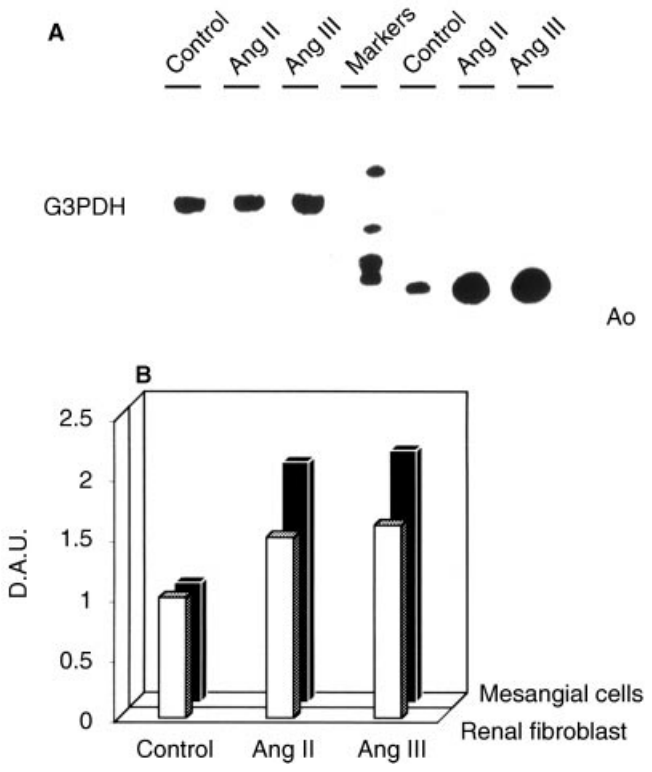

Fig. 3. Ang III up-regulates angiotensinogen gene expression in renal cells. Mesangial cells and renal interstitial fibroblasts constitutively express angiotensinogen (Ao) mRNA as shown by RT-PCR. (*A*) Cells were stimulated for six hours with 10^{-7} M Ang III and Ang II. A representative experiment of RT-PCR of three is shown; G3PDH was used as internal control. (*B*) Densitometric analysis of the PCR products. Values are expressed as *N*-fold of increase versus control, normalized to the respective G3PDH.

DISCUSSION

Among the peptides of RAS, only Ang II has been considered as a renal growth factor [1, 2 and the references therein]. Although Ang III, the N terminal deleted heptapeptide fragment of Ang II, presents some biological functions, its role in the events associated to progressive renal diseases has not been evaluated. It is well known that Ang II activates some short and long growth-related responses, such as induction of early response genes and cell proliferation [1, 2]. The effect of Ang II on cell growth is controversial. In mesangial and vascular smooth muscle cells, both a hyperplasic or hypertrophic response has been found [reviewed in 1]. In renal interstitial fibroblasts, we have recently reported that Ang II increases cell proliferation [12], while in proximal tubular cells it induces cell hypertrophy [2]. Nevertheless, those stimulatory effects were mediated by AT_1 receptors, as shown by the employment of Dup753, a specific nonpeptidic receptor antagonist [1, 2, 12]. In all of these cell types as well as in *in vivo* infusion, Ang II induces c-*fos* mRNA expression [1, 2, 12]. The c-*fos* protein acts as a transcriptional factor that mediates growth response [18]. We have observed that after one hour of stimulation Ang III induces c-*fos* gene expression with an intensity similar to Ang II. Although future studies are necessary, these results suggest that Ang III could participate in the control of cell proliferation.

One feature of renal disease is the accumulation of extracellular matrix production in the glomerulus and interstitium [10]. *In vitro* and *in vivo* studies have demonstrated that local Ang II could contribute to these phenomena [10–16]. Ang II activates mesangial cells, tubular cells and, as we have recently shown, renal interstitial fibroblasts increasing the expression and synthesis of matrix proteins [1, 2, 12]. These effects seem to be mediated by the release of transforming growth factor-β (TGF-β) [2]. In experimental models of renal damage associated to elevated renal Ang II production, an upregulation in renal TGF-β expression was noted in correlation with increased extracellular matrix protein mRNA expression and deposition, that diminished in response to ACE inhibition and AT_1 receptor blockade treatment [14–17]. In those settings, an increase in general and specific proteases has been described [3, 8, 9], suggesting the presence of Ang II degradation products, such as Ang III that could eventually contribute to amplify the renal damage. Our studies have shown that in cultured mesangial cells and renal interstitial fibroblasts Ang III increased TGF-β mRNA expression and fibronectin production, suggesting that this peptide could also contribute to the matrix accumulation observed during renal damage.

Several studies have demonstrated the existence of an independent tissue RAS in several organs, including the kidney. During renal injury, an activation of renal RAS and an increase of local Ang II generation have been observed in situations associated or not to hypertension [23–25]. In this sense, in a normotensive model of immune complex nephritis, we have described an increase in renal ACE activity and upregulation of renal angiotensinogen mRNA expression correlated with proteinuria and sclerosis [25]. In addition, we have observed that in cultured mesangial cells and renal interstitial fibroblasts angiotensinogen mRNA expression was up-regulated in response to Ang II and Ang III, suggesting that these peptides could contribute to the increased local angiotensin generation, through angiotensinogen gene regulation. The expression of this gene is controlled mainly by two transcription factor families, NF-κB and CCAAT/enhancer binding protein, which bind an inducible enhancer in the angiotensinogen gene promoter [22]. Recent data have shown activation of local NF-κB during tissue damage, including the kidney, that diminished in response to ACE inhibition [13, 26]. Moreover, in cultured mesangial cells Ang II activates NF-κB [13]. These data suggest that activation of NF-κB might also contribute to a sustained synthesis of local angiotensins, through angiotensinogen gene expression, and therefore to a potential further tissue damage.

In summary, our data show that in cultured mesangial

cells and renal interstitial fibroblasts Ang III elicits overexpression of genes involved in renal damage, regulating cell growth and matrix production. These results support the hypothesis that Ang II is not the one and only effector peptide of the RAS, and afford more information to modify our classical view of this system.

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